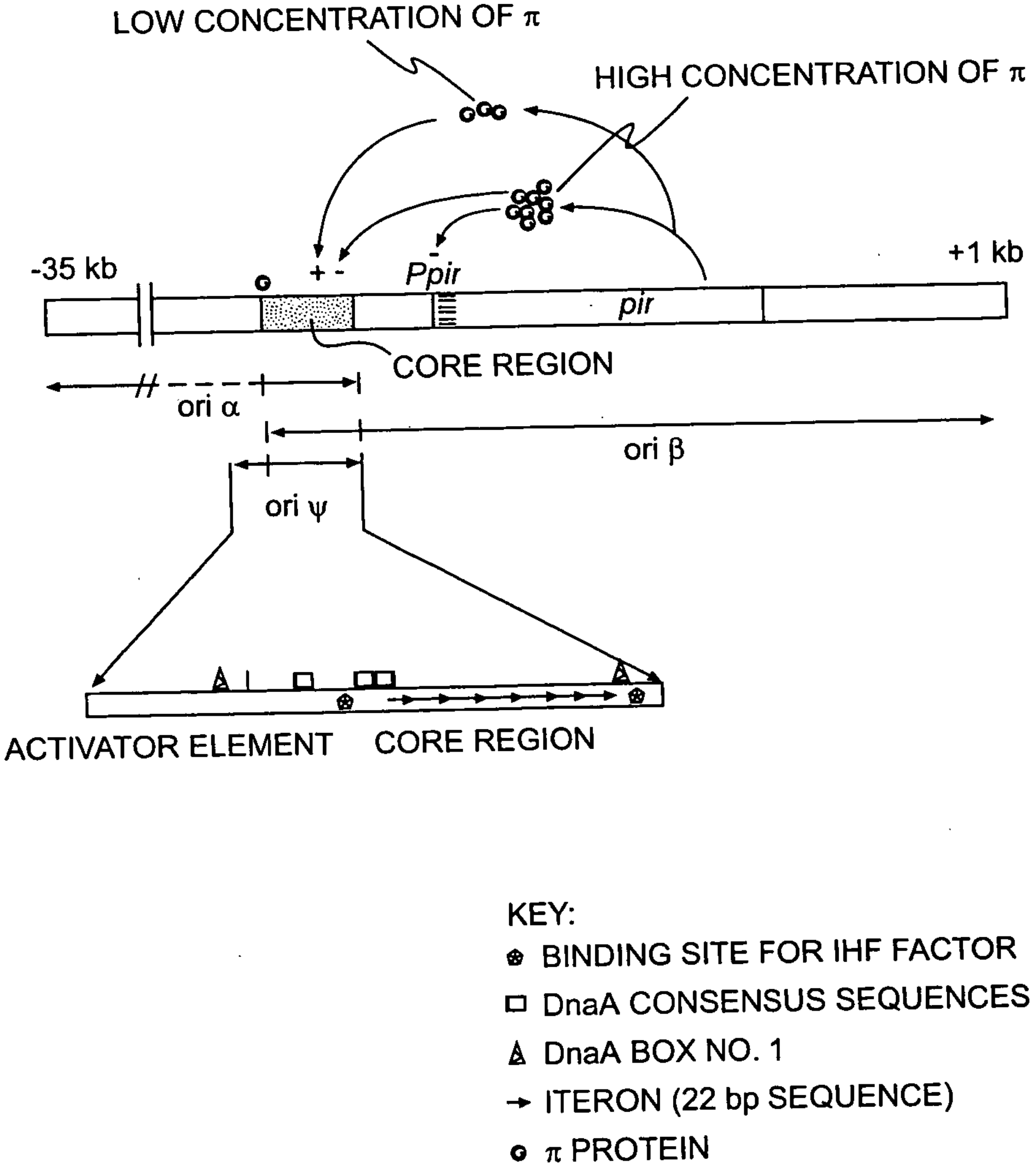

LOW CONCENTRATION OF π
HIGH CONCENTRATION OF π
-35 kb
+ -
-
Ppir
+1 kb
pir
CORE REGION
ori α
ori β
ori ψ
ACTIVATOR ELEMENT
CORE REGION
KEY:
⬟ BINDING SITE FOR IHF FACTOR
□ DnaA CONSENSUS SEQUENCES
▲ DnaA BOX NO. 1
→ ITERON (22 bp SEQUENCE)
○ π PROTEIN

FIG. 1

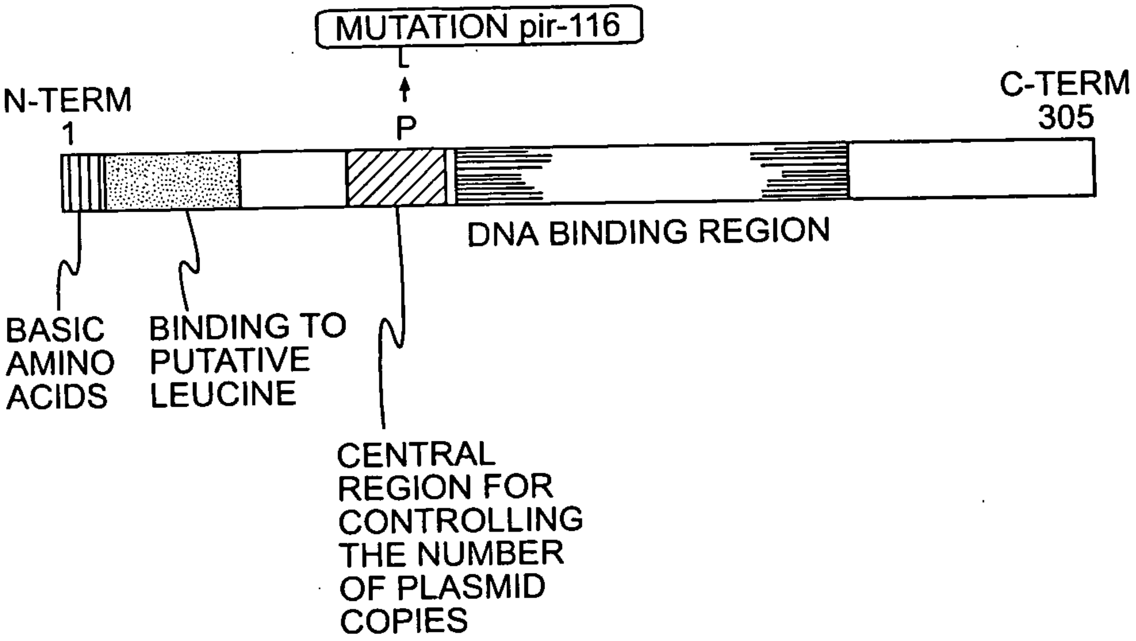

MUTATION pir-116
L
P
N-TERM
1
C-TERM
305
DNA BINDING REGION
BASIC AMINO ACIDS
BINDING TO PUTATIVE LEUCINE
CENTRAL REGION FOR CONTROLLING THE NUMBER OF PLASMID COPIES

FIG. 2

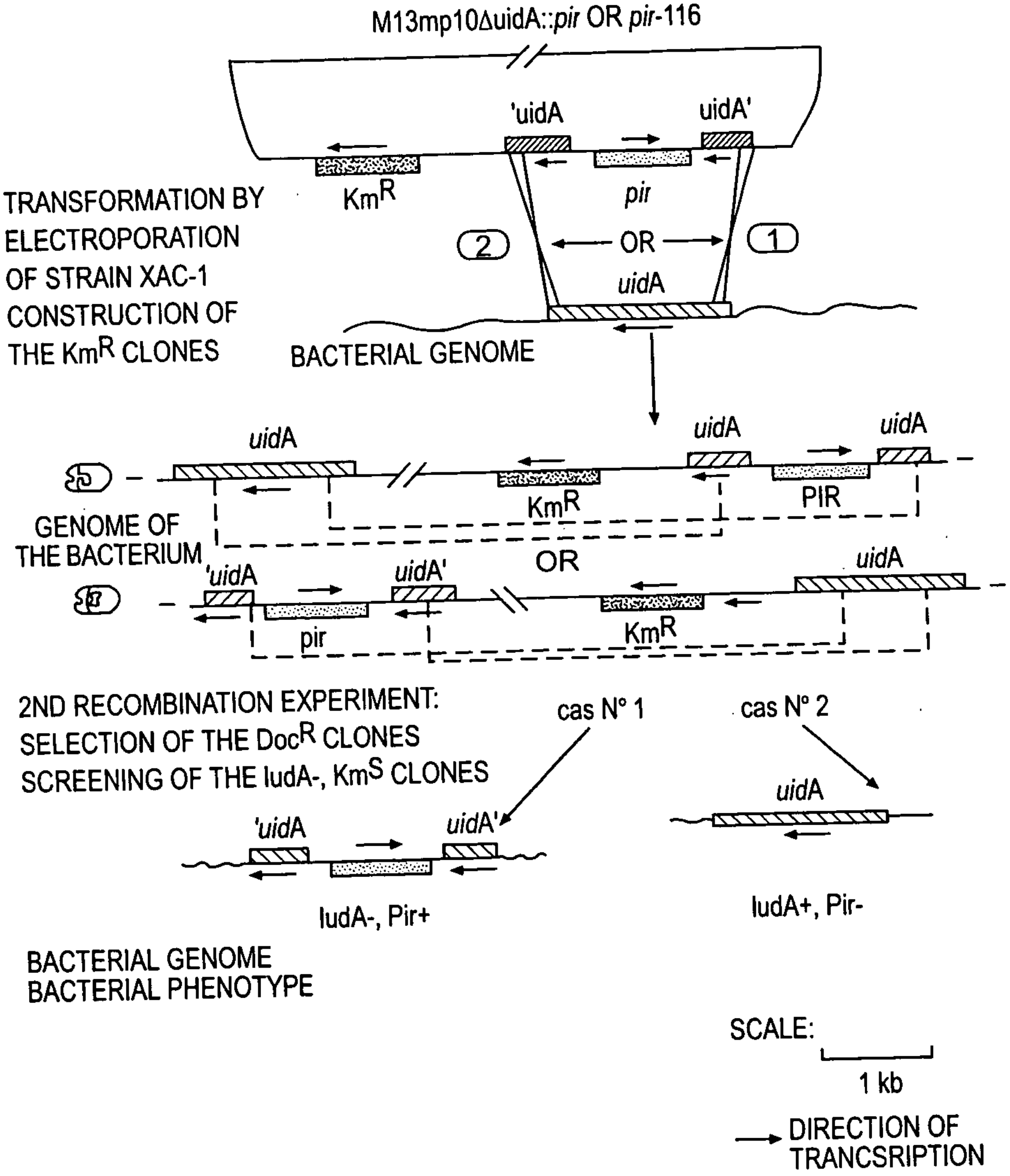

M13mp10ΔuidA::pir OR pir-116
'uidA
uidA'
KmR
pir
TRANSFORMATION BY ELECTROPORATION OF STRAIN XAC-1 CONSTRUCTION OF THE KmR CLONES
2
OR
1
uidA
BACTERIAL GENOME
uidA
uidA
uidA
KmR
PIR
GENOME OF THE BACTERIUM
OR
'uidA
uidA'
uidA
pir
KmR
2ND RECOMBINATION EXPERIMENT: SELECTION OF THE DocR CLONES SCREENING OF THE ludA-, KmS CLONES
cas N° 1
cas N° 2
'uidA
uidA'
uidA
ludA-, Pir+
ludA+, Pir-
BACTERIAL GENOME
BACTERIAL PHENOTYPE
SCALE:
1 kb
DIRECTION OF TRANCSRIPTION

FIG. 3

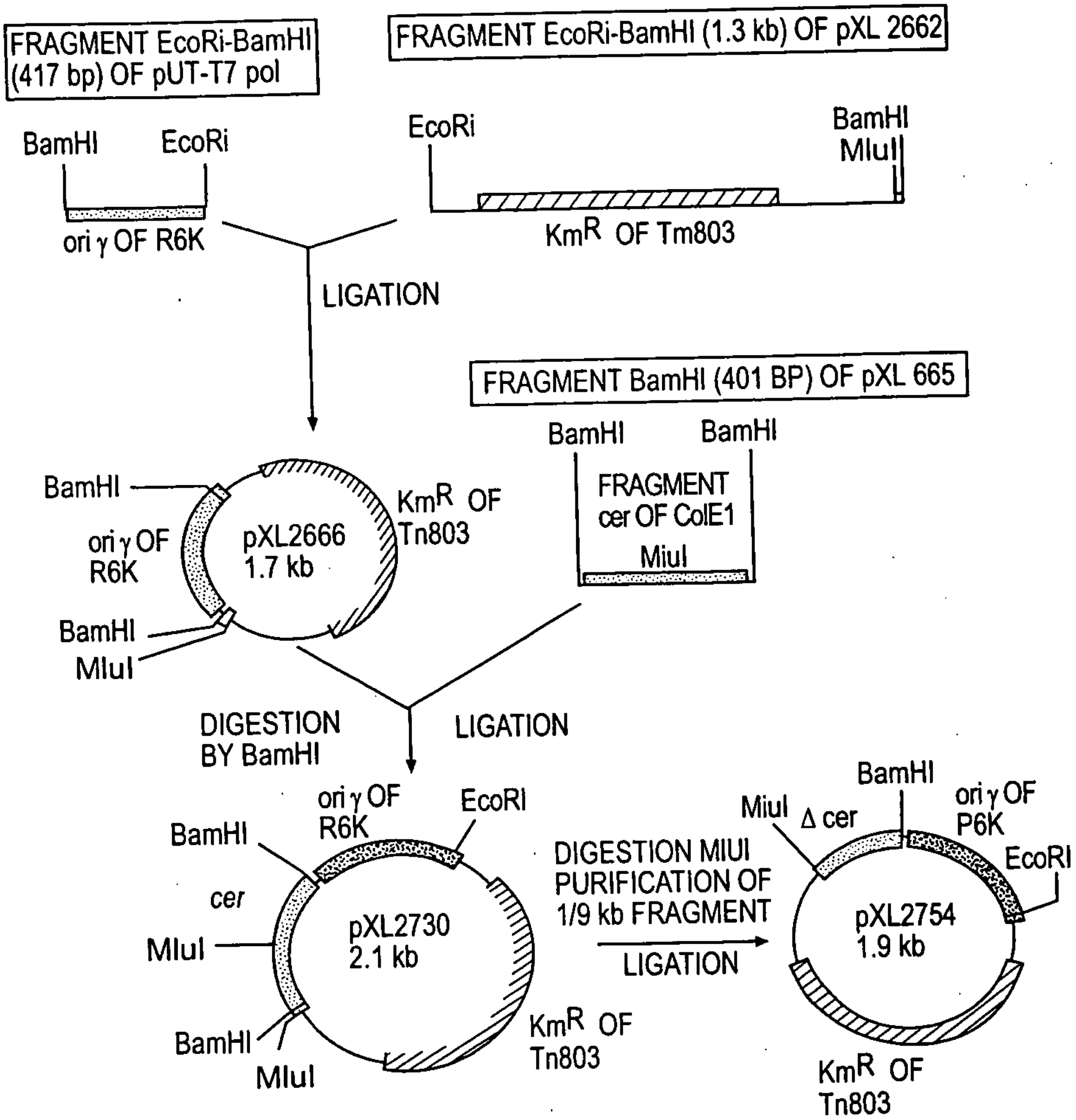

FRAGMENT EcoRi-BamHI (417 bp) OF pUT-T7 pol
FRAGMENT EcoRi-BamHI (1.3 kb) OF pXL 2662
BamHI
EcoRi
ori γ OF R6K
EcoRi
BamHI
MluI
Km^R OF Tm803
LIGATION
FRAGMENT BamHI (401 BP) OF pXL 665
BamHI
BamHI
FRAGMENT cer OF ColE1
MiuI
BamHI
ori γ OF R6K
pXL2666 1.7 kb
Km^R OF Tn803
BamHI
MluI
DIGESTION BY BamHI
LIGATION
ori γ OF R6K
EcoRI
BamHI
cer
MluI
pXL2730 2.1 kb
BamHI
MluI
Km^R OF Tn803
DIGESTION MIUI PURIFICATION OF 1/9 kb FRAGMENT
LIGATION
BamHI
MiuI
Δ cer
ori γ OF P6K
EcoRI
pXL2754 1.9 kb
Km^R OF Tn803

FIG. 4

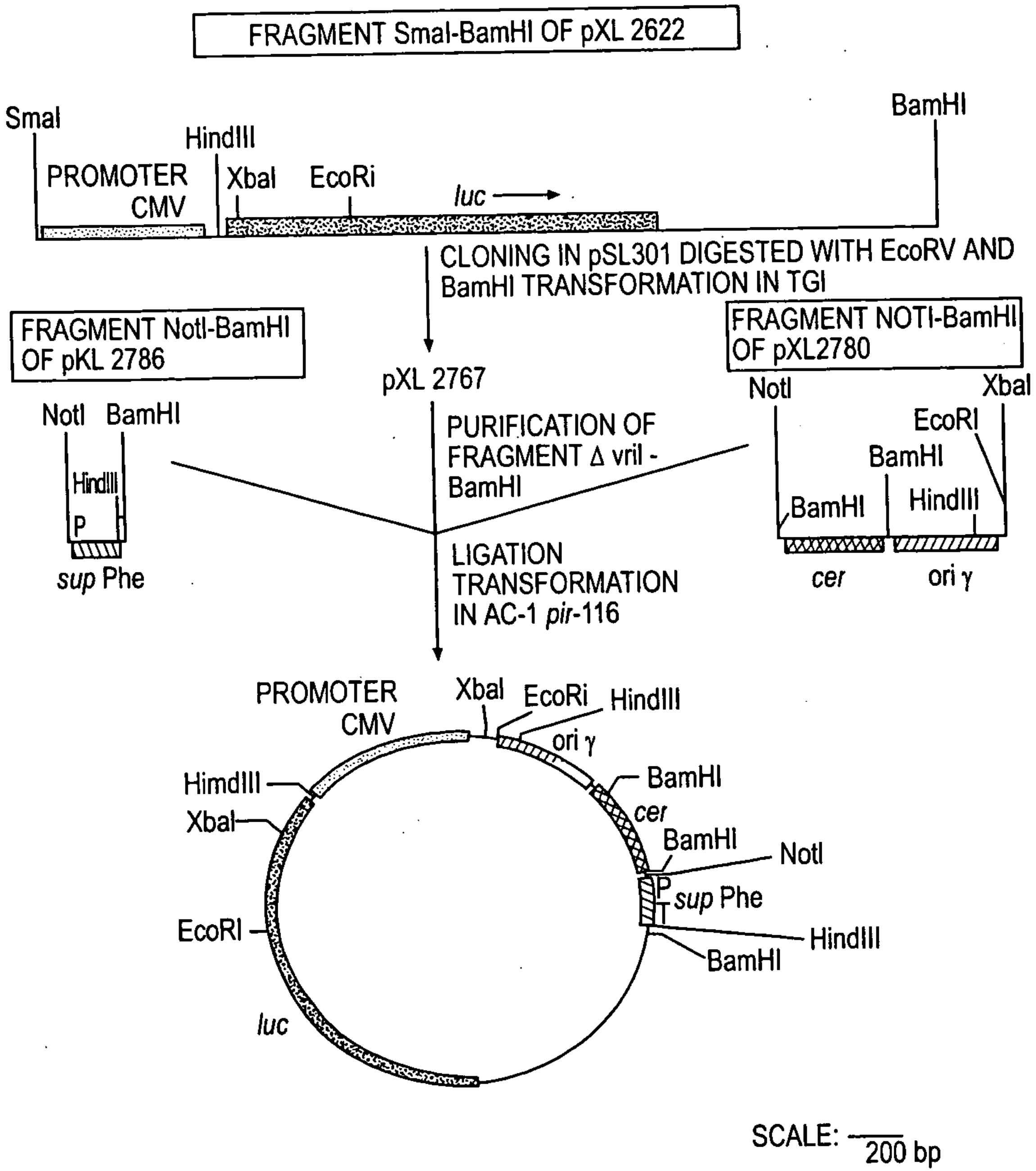

FRAGMENT SmaI-BamHI OF pXL 2622
SmaI
HindIII
PROMOTER CMV
XbaI
EcoRi
luc
BamHI
CLONING IN pSL301 DIGESTED WITH EcoRV AND BamHI TRANSFORMATION IN TGI
FRAGMENT NotI-BamHI OF pKL 2786
pXL 2767
FRAGMENT NOTI-BamHI OF pXL2780
NotI
BamHI
HindIII
P
sup Phe
PURIFICATION OF FRAGMENT Δ vriI - BamHI
NotI
XbaI
EcoRI
BamHI
BamHI
HindIII
cer
ori γ
LIGATION TRANSFORMATION IN AC-1 pir-116
PROMOTER CMV
XbaI
EcoRi
HindIII
ori γ
BamHI
cer
BamHI
NotI
HimdIII
XbaI
EcoRI
P
sup Phe
T
HindIII
BamHI
luc
SCALE: 200 bp
P: PROMOTER P_{ipp}
T: TERMINATOR T_{paC}

FIG. 5

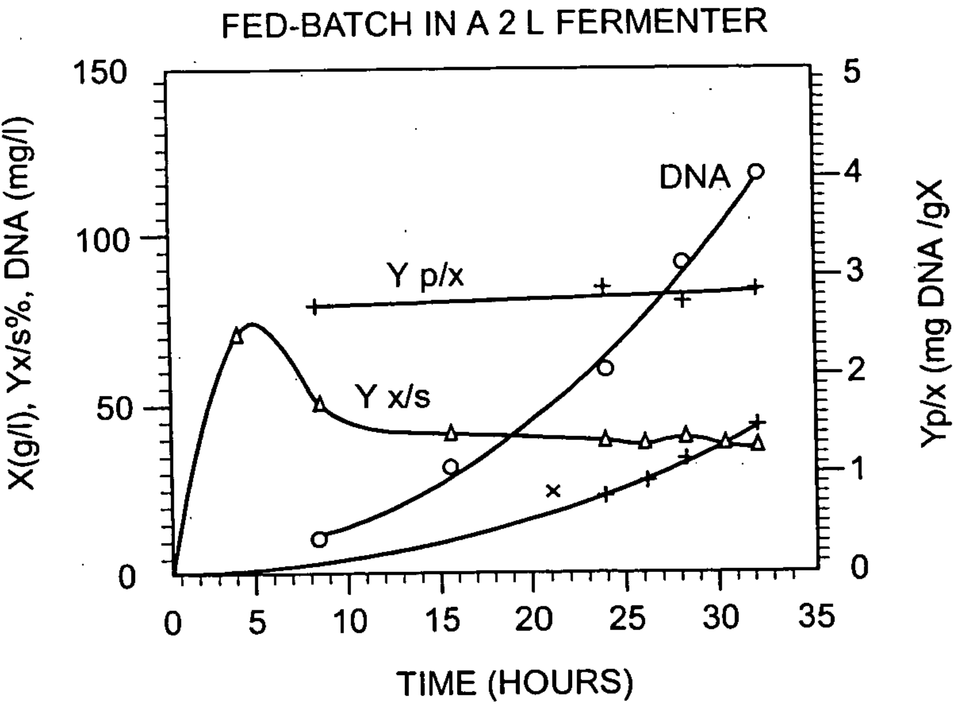
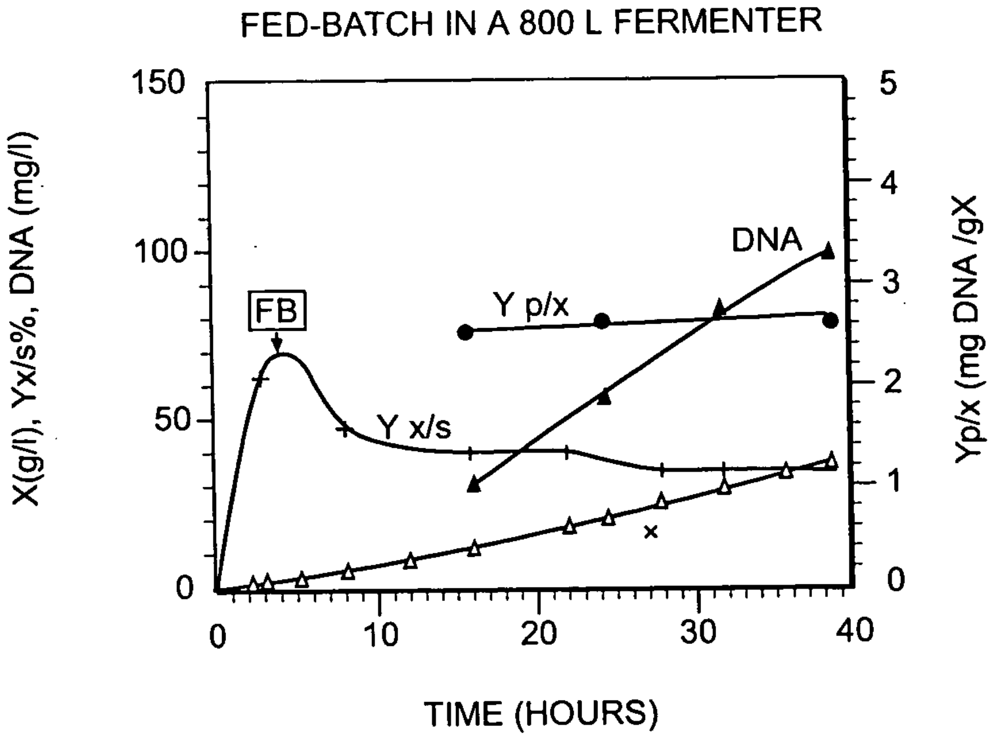

FED-BATCH IN A 2 L FERMENTER
150
100
50
0
X(g/l), Yx/s%, DNA (mg/l)
5
4
3
2
1
0
Yp/x (mg DNA /gX
DNA
Y p/x
Y x/s
0
5
10
15
20
25
30
35
TIME (HOURS)

FIG. 6



FED-BATCH IN A 800 L FERMENTER
X(g/l), Yx/s%, DNA (mg/l)
Yp/x (mg DNA /gX
TIME (HOURS)
150
100
50
0
5
4
3
2
1
0
0
10
20
30
40
FB
Y x/s
Y p/x
DNA

FIG. 7

CONSTRUCTION OF THE EXPRESSION VECTOR pXL3056

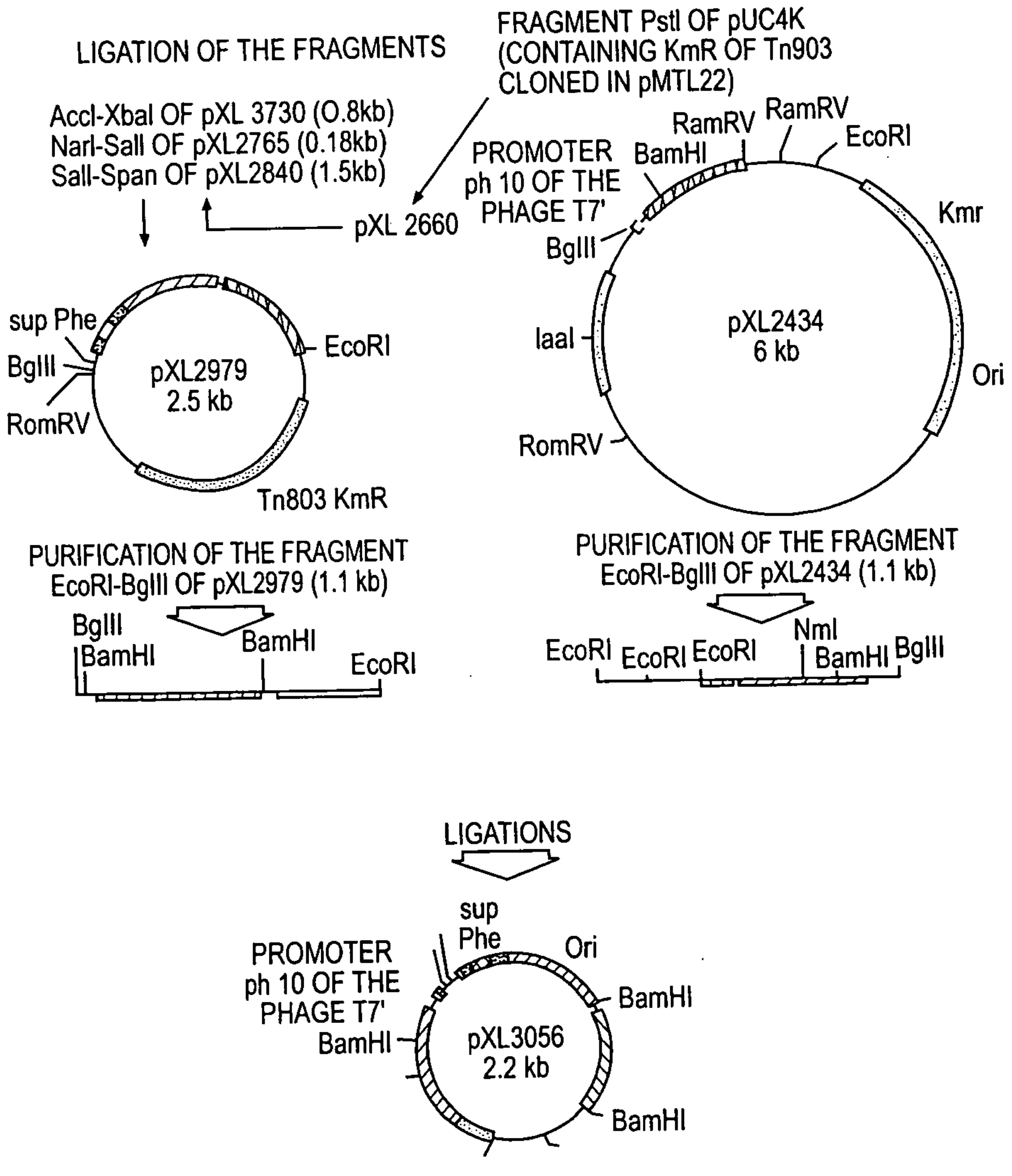


FIG. 8

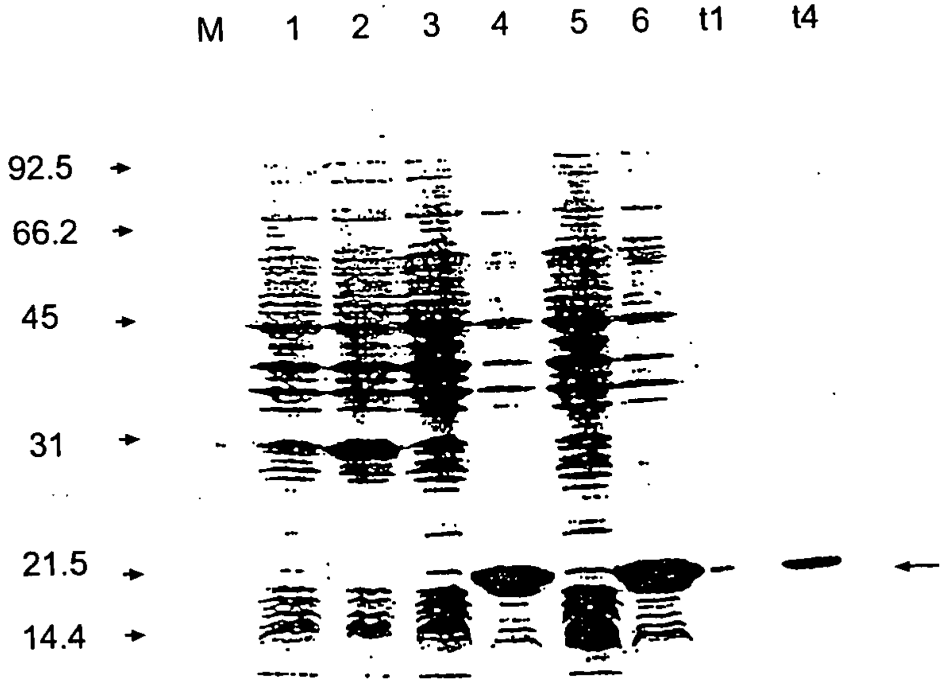


FIG. 9

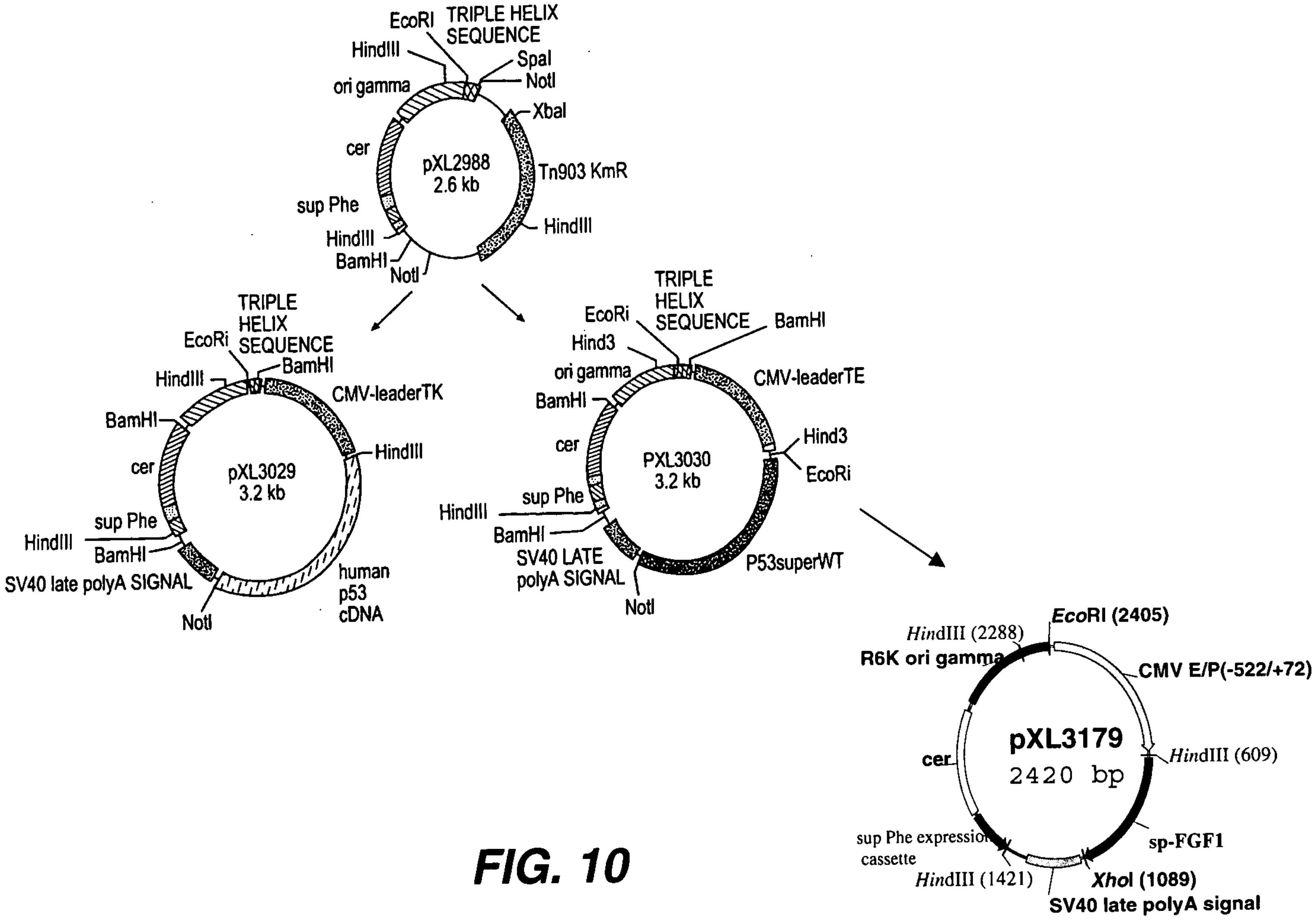

EcoRI
TRIPLE HELIX SEQUENCE
HindIII
SpaI
NotI
ori gamma
XbaI
cer
pXL2988
2.6 kb
Tn903 KmR
sup Phe
HindIII
HindIII
BamHI
NotI
TRIPLE HELIX SEQUENCE
EcoRi
BamHI
HindIII
CMV-leaderTK
BamHI
HindIII
cer
pXL3029
3.2 kb
sup Phe
HindIII
BamHI
SV40 late polyA SIGNAL
human p53 cDNA
NotI
TRIPLE HELIX SEQUENCE
EcoRi
BamHI
Hind3
ori gamma
CMV-leaderTE
BamHI
Hind3
cer
PXL3030
3.2 kb
EcoRi
sup Phe
HindIII
BamHI
SV40 LATE polyA SIGNAL
P53superWT
NotI
EcoRI (2405)
HindIII (2288)
R6K ori gamma
CMV E/P(-522/+72)
pXL3179
2420 bp
cer
HindIII (609)
sup Phe expression cassette
sp-FGF1
HindIII (1421)
XhoI (1089)
SV40 late polyA signal

FIG. 10

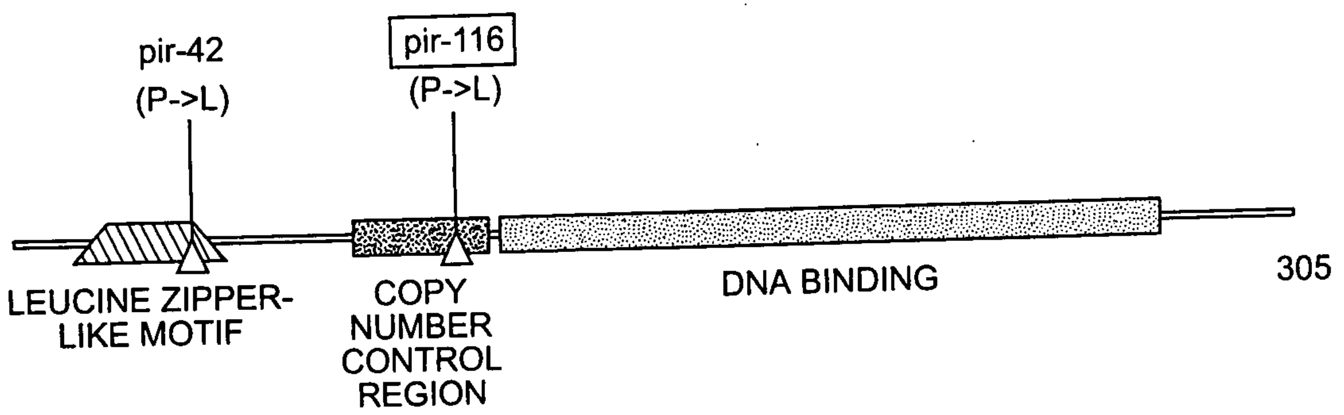

pir-42
(P->L)
pir-116
(P->L)
LEUCINE ZIPPER-LIKE MOTIF
COPY NUMBER CONTROL REGION
DNA BINDING
305

FIG. 11

(SEQ ID NO: 21)

Met Arg Leu Lys Val Met Met Asp Val Asn Lys Lys Thr Lys Ile Arg His Arg
1 ATG AGA CTC AAG GTC ATG ATG GAC GTG AAC AAA AAA ACG AAA ATT CGC CAC CGA

Asn Glu Leu Asn His Thr Leu Ala Gln Leu Pro Leu Pro Ala Lys Arg Val Met
55 AAC GAG CTA AAT CAC ACC CTG GCT CAA CTT CCT TTG CCC GCA AAG CGA GTG ATG

Tyr Met Ala Leu Ala Leu Ile Asp Ser Lys Glu Pro Leu Glu Arg Gly Arg Val
109 TAT ATG CGC CTT CGT CTC ATC GAT AGC AAA GAA CCT CTT GAA CGA GGG CGA GTT

Phe Lys Ile Arg Ala Glu Asp Leu Ala Ala Leu Ala Lys Ile Thr Pro Ser Leu
163 TTC AAA ATT AGG GCT GAA GAC CTT GCA GCG CTC GCC AAA ATC ACC CCA TCG CTT

Ala Tyr Arg Gln Leu Lys Glu Gly Gly Lys Leu Lue Gly Ala Ser Lys Ile Ser
217 GCT TAT CGA CAA TTA AAA GAG GGT GGT AAA TTA CTT GGT GCC AGC AAA ATT TCG

Leu Arg Gly Asp Asp Ile Ile Ala Leu Ala Lys Glu Leu Asn Leu Leu Phe Thr
271 CTA AGA GGG GAT GAT ATC ATT GCT TTA GCT AAA GAG CTT AAC CTG CTC TTT ACT

Ala Lys Asn Ser Pro Glu Glu Leu Asp Leu Asn Ile Ile Glu Trp Ile Ala Tyr
325 GCT AAA AAC TCC CCT GAA GAG TTA GAT CTT AAC ATT ATT GAG TGG ATA GCT TAT

Ser Asn Asp Glu Gly Tyr Leu Ser Leu Lys Phe Thr Arg Thr Ile Glu Pro Tyr
379 TCA AAT GAT GAA GGA TAC TTG TCT TTA AAA TTC ACC AGA ACC ATA GAA CCA TAT

Ile Ser Ser Leu Ile Gly Lys Lys Asn Lys Phe Thr Thr Gln Leu Leu Thr Ala
433 ATC TCT AGC CTT ATT GGG AAA AAA AAT AAA TTC ACA ACG CAA TTG TTA ACG GCA

Ser Leu Arg Leu Ser Ser Gln Tyr Ser Ser Ser Leu Tyr Gln Leu Ile Arg Lys
487 AGC TTA CGC TTA AGT AGC CAG TAT TCA TCT TCT CTT TAT CAA CTT ATC AGG AAG

His Tyr Ser Asn Phe Lys Lys Lys Asn Tyr Phe Ile Ile Ser Val Asp Glu Leu
541 CAT TAC TCT AAT TTT AAG AAG AAA AAT TAT TTT ATT ATT TCC GTT GAT GAG TTA

FIG. 12.1

```
    Lys Glu Glu Leu Ile Als Yyr Thr Phe Asp Lys Asp Gly Asn Ile Glu Tyr Lys
595 AAG GAA GAG TTA ATA GCT TAT ACT TTT GAT AAA GAT GGA AAT ATT GAG TAC AAA

    Tyr Pro Asp Phe Pro Ile Phe Lys Arg Asp Val Leu Asn Lys Ala Ile Ala Glu
649 TAC CCT GAC TTT CCT ATT TTT AAA AGG GAT GTG TTA AAT AAA GCC ATT GCT GAA

    Ile Lys Lys Lys Thr Glu Ile Ser Phe Val Gly Phe Thr Val His Glu Lys Glu
703 ATT AAA AAG AAA ACA GAA ATA TCG TTT GTT GGC TTC ACT GTT CAT GAA AAA GAA

    Gly Arg Lys Ile Ser Lys Leu Lys Phe Glu Phe Val Val Asp Glu Asp Glu Phe
757 GGA AGA AAA ATT AGT AAG CTG AAG TTC GAA TTT GTC GTT GAT GAA GAT GAA TTT

    Ser Gly Asp Lys Asp Asp Glu Ala Phe Phe Met Asn Leu Ser Glu Ala Asp Ala
811 TCT GGC GAT AAA GAT GAT GAA GCT TTT TTT ATG AAT TTA TCT GAA GCT GAT GCA

    Ala Phe Leu Lys Val Phe Asp Glu Thr Val Pro Pro Lys Lys Ala Lys Gly ***
865 GCT TTT CTC AAG GTA TTT GAT GAA ACC GTA CCT CCC AAA AAA GCT AAG GGG TGA
```

FIG. 12.2

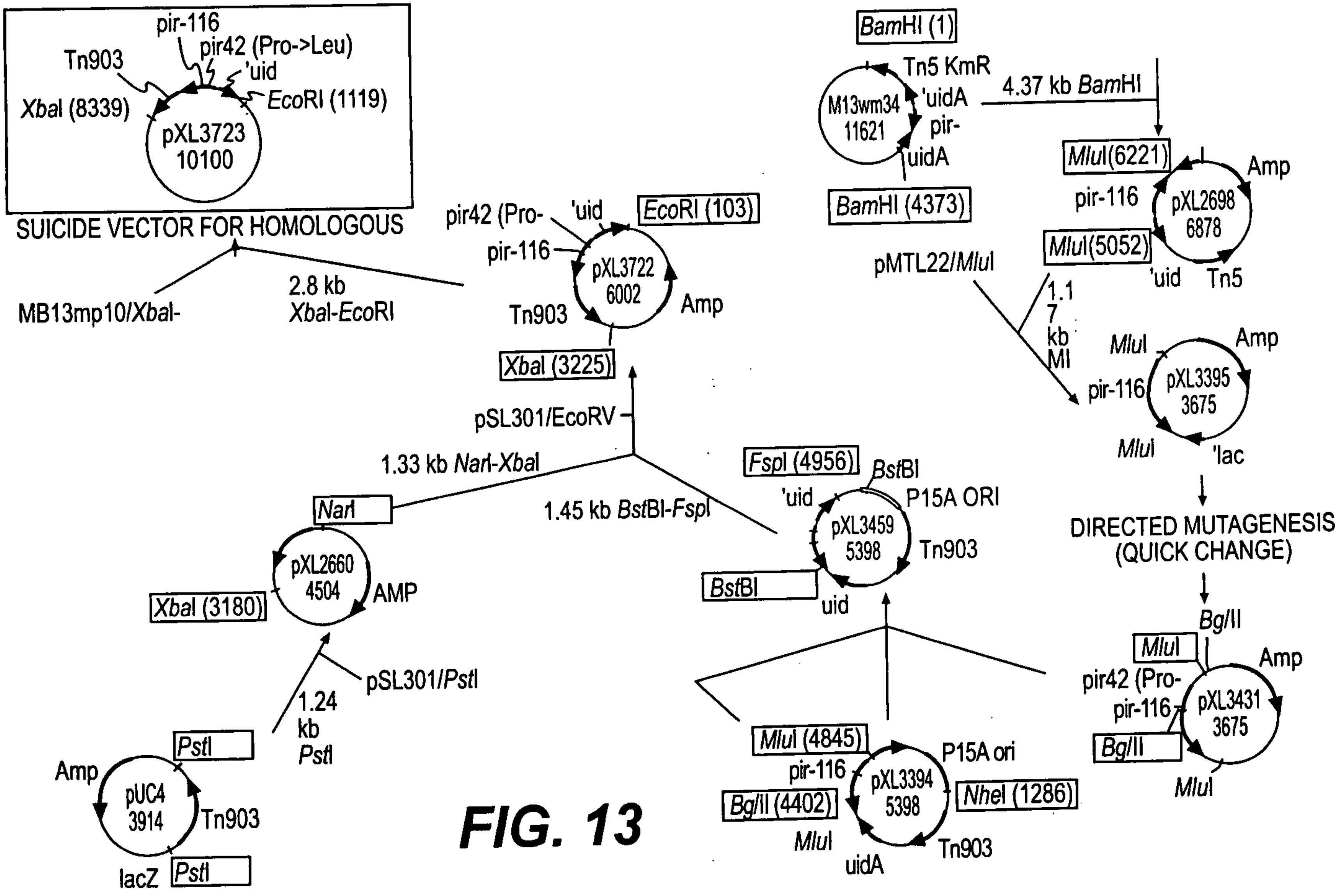
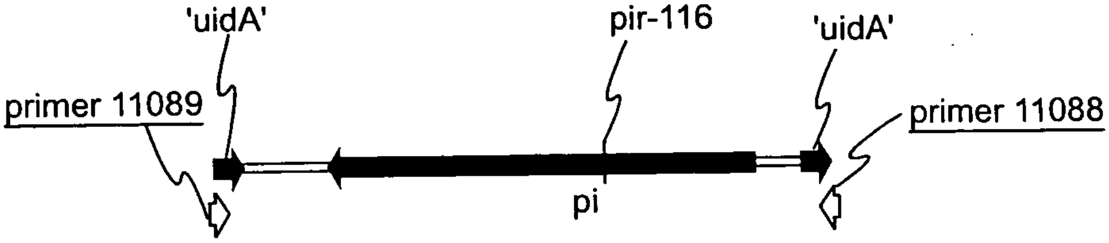
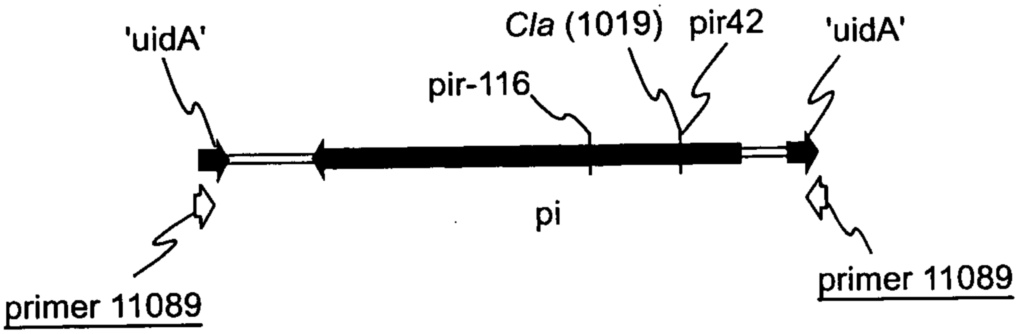

pir-116
pir42 (Pro->Leu)
Tn903
'uid
XbaI (8339)
EcoRI (1119)
pXL3723
10100
SUICIDE VECTOR FOR HOMOLOGOUS
MB13mp10/XbaI-
2.8 kb
XbaI-EcoRI
pir42 (Pro-
'uid
EcoRI (103)
pir-116
pXL3722
6002
Tn903
Amp
XbaI (3225)
pSL301/EcoRV
1.33 kb NarI-XbaI
1.45 kb BstBI-FspI
NarI
pXL2660
4504
XbaI (3180)
AMP
pSL301/PstI
1.24
kb
PstI
PstI
Amp
pUC4
3914
Tn903
lacZ
PstI
BamHI (1)
Tn5 KmR
M13wm34
11621
'uidA
pir-
uidA
BamHI (4373)
4.37 kb BamHI
MluI(6221)
Amp
pir-116
pXL2698
6878
MluI(5052)
'uid
Tn5
pMTL22/MluI
1.1
7
kb
MI
MluI
Amp
pir-116
pXL3395
3675
MluI
'lac
DIRECTED MUTAGENESIS
(QUICK CHANGE)
BglII
MluI
Amp
pir42 (Pro-
pir-116
pXL3431
3675
BglII
MluI
FspI (4956)
BstBI
'uid
P15A ORI
pXL3459
5398
Tn903
BstBI
uid
MluI (4845)
P15A ori
pir-116
pXL3394
5398
BglII (4402)
NheI (1286)
MluI
uidA
Tn903

FIG. 13



PCR pir116 (11089-11088)

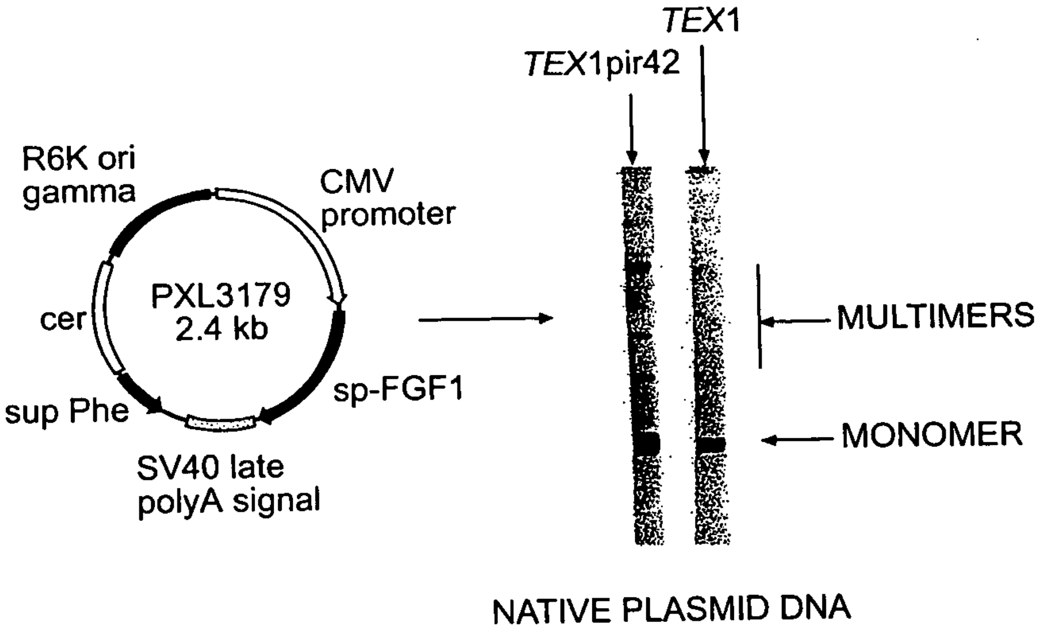
1304 bp



PCR pir116-pir42 (11089-11088)

1304 bp

FIG. 14



R6K ori gamma
CMV promoter
PXL3179
2.4 kb
cer
sp-FGF1
sup Phe
SV40 late polyA signal
TEX1
TEX1pir42
MULTIMERS
MONOMER
NATIVE PLASMID DNA

FIG. 15

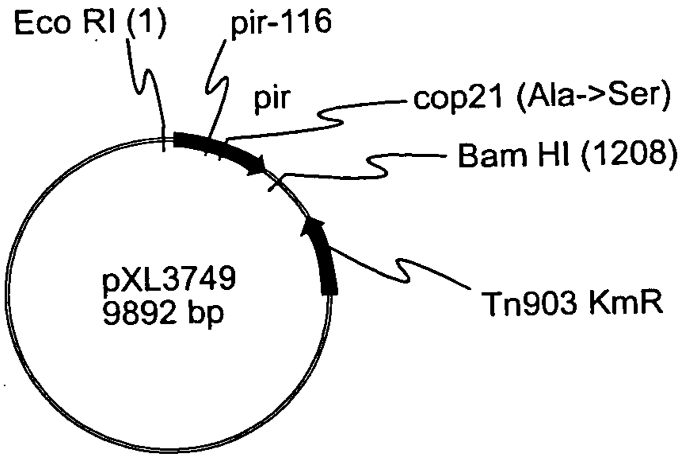
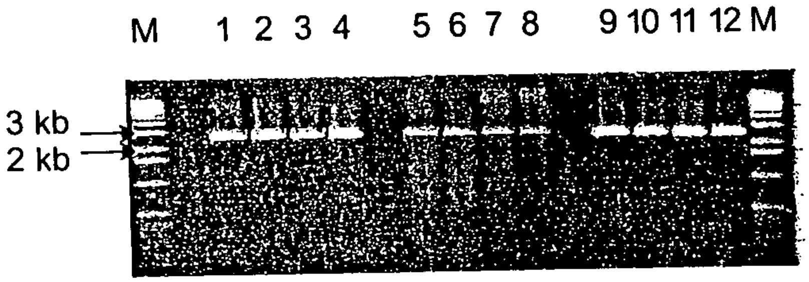

Eco RI (1)
pir-116
pir
cop21 (Ala->Ser)
Bam HI (1208)
Tn903 KmR
pXL3749
9892 bp

FIG. 16



M: 1 kb LADDER (LIFE BIOTECHNOLOGIES)
1->4: TEX1*cop*21 (pXL2979)
5->8: XAC1*pir* (pXL2979)
9->12: TEX1 (pXL2979)

FIG. 17

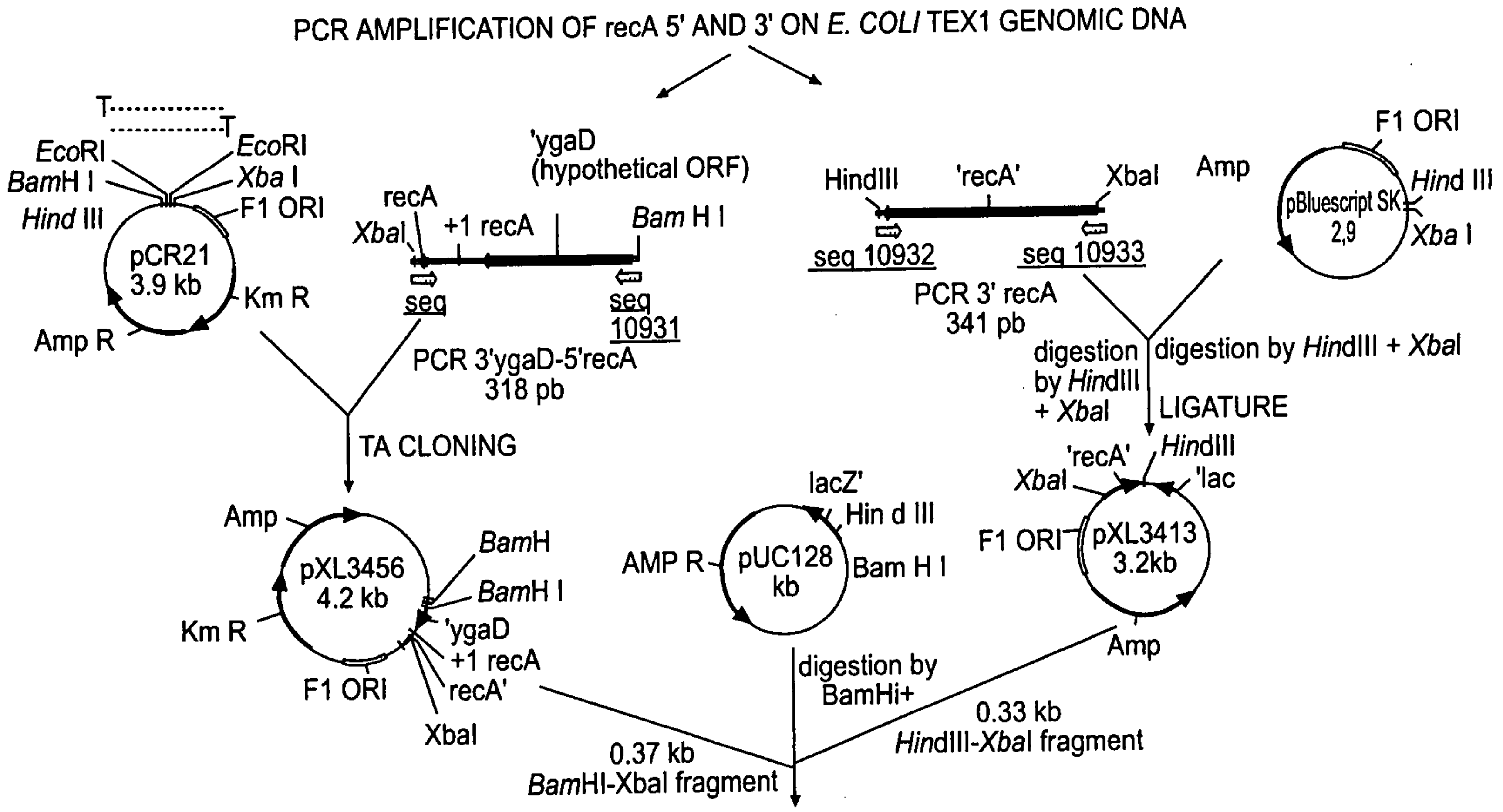

PCR AMPLIFICATION OF recA 5' AND 3' ON E. COLI TEX1 GENOMIC DNA
T
T
EcoRI
BamH I
Hind III
EcoRI
Xba I
F1 ORI
pCR21
3.9 kb
Km R
Amp R
'ygaD
(hypothetical ORF)
recA
XbaI
+1 recA
Bam H I
seq
seq
10931
PCR 3'ygaD-5'recA
318 pb
TA CLONING
Amp
pXL3456
4.2 kb
Km R
F1 ORI
BamH
BamH I
'ygaD
+1 recA
recA'
XbaI
HindIII
'recA'
XbaI
seq 10932
seq 10933
PCR 3' recA
341 pb
Amp
F1 ORI
pBluescript SK
2,9
Hind III
Xba I
digestion by HindIII + XbaI
digestion by HindIII + XbaI
LIGATURE
'recA'
HindIII
XbaI
'lac
F1 ORI
pXL3413
3.2kb
Amp
lacZ'
Hin d III
AMP R
pUC128
kb
Bam H I
digestion by BamHi+
0.37 kb
BamHI-XbaI fragment
0.33 kb
HindIII-XbaI fragment

FIG. 18.1

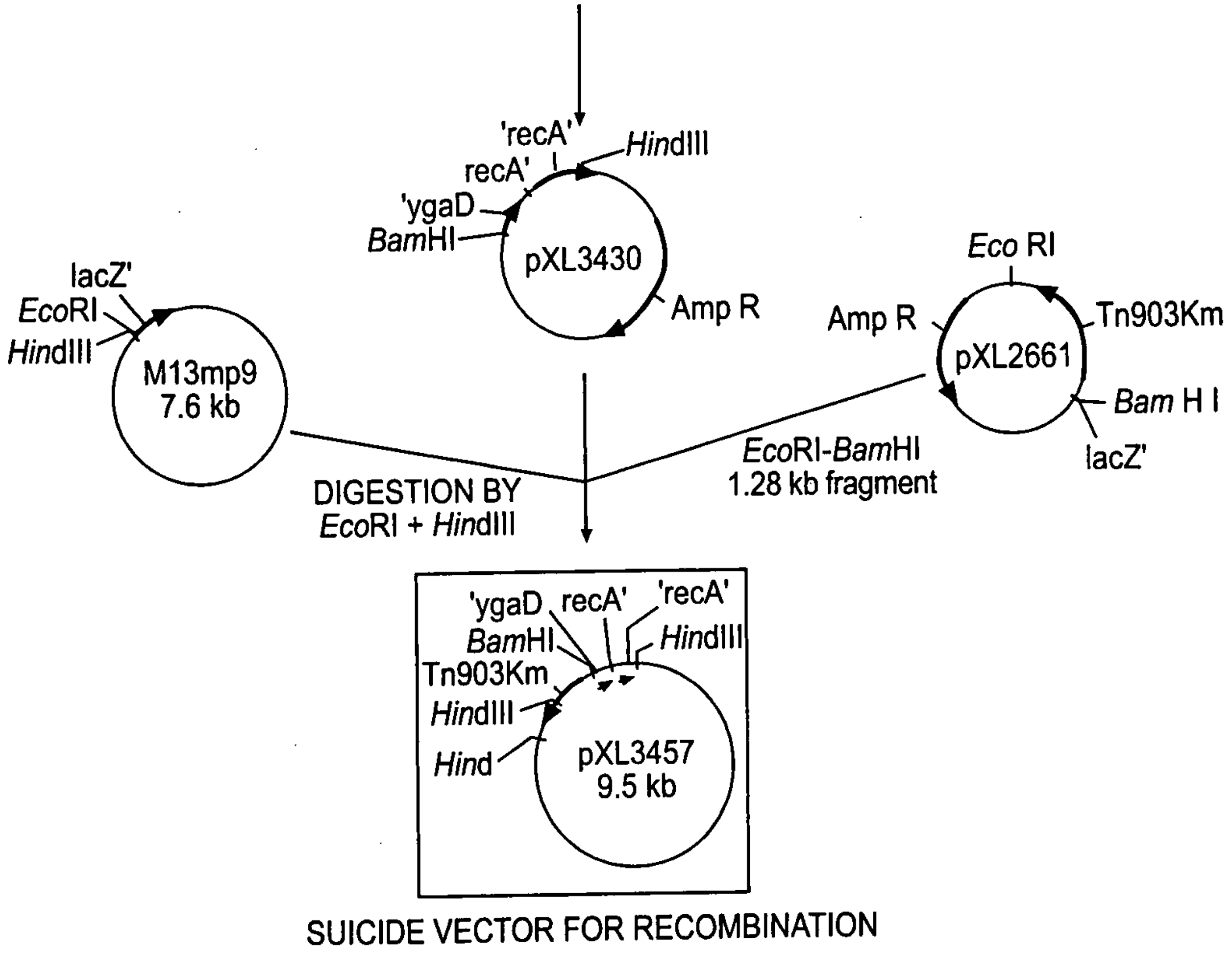

'recA'
recA'
HindIII
'ygaD
BamHI
pXL3430
Amp R
lacZ'
EcoRI
HindIII
M13mp9
7.6 kb
Eco RI
Amp R
Tn903Km
pXL2661
Bam H I
lacZ'
EcoRI-BamHI
1.28 kb fragment
DIGESTION BY
EcoRI + HindIII
'ygaD
recA'
'recA'
BamHI
HindIII
Tn903Km
HindIII
Hind
pXL3457
9.5 kb
SUICIDE VECTOR FOR RECOMBINATION

FIG. 18.2

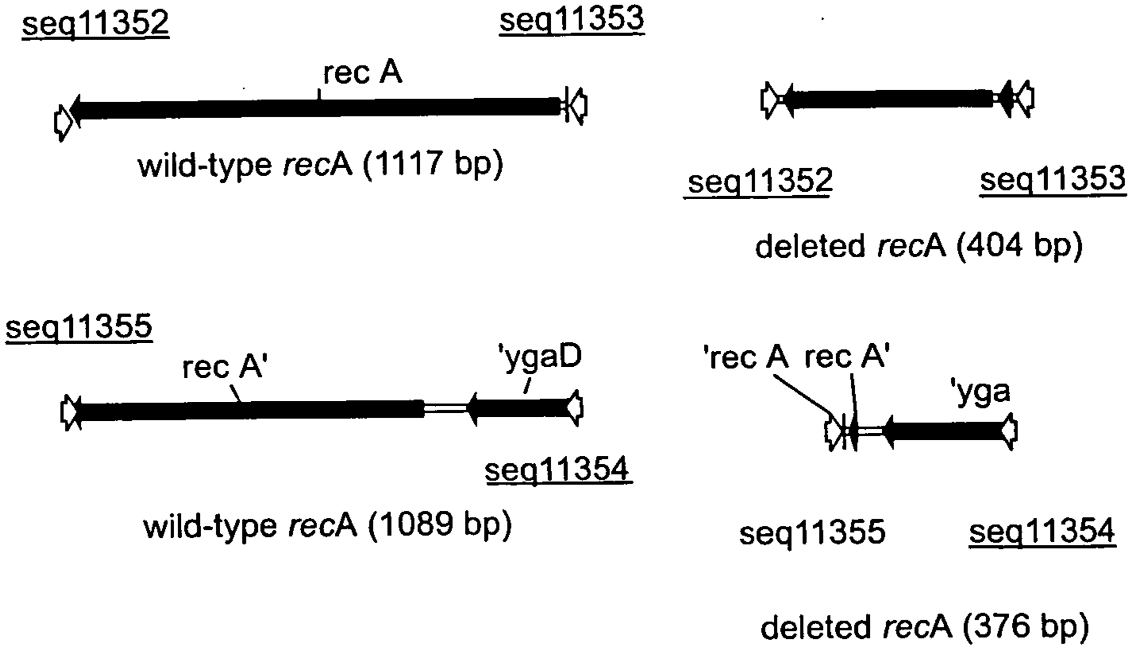
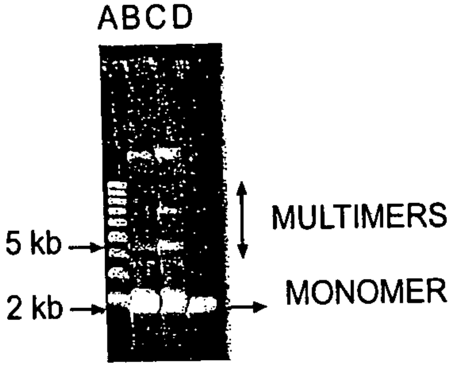

seq11352
seq11353
rec A
wild-type recA (1117 bp)
seq11352
seq11353
deleted recA (404 bp)
seq11355
rec A'
'ygaD
seq11354
wild-type recA (1089 bp)
'rec A
rec A'
'yga
seq11355
seq11354
deleted recA (376 bp)

FIG. 19



A: SUPERCOILED DNA LADDER (PROMEGA)
B: pXL3179 IN TEX2 *pir*42
C: pXL3179 IN TEX1 *pir*42
D: pXL3179 IN TEX1

FIG. 20

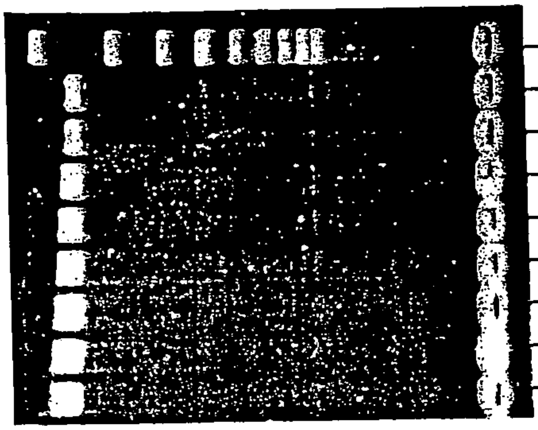

SUPERCOILED DNA LADDER (2,3 . . . Kb)
Op1328S5 18h51
Op1328S5 18h51
Op1328S5 25h30
Op1328S5 25h30
Op1328S5 43h
Op1328S5 43h
Op1328S5 48h47 (FINAL)
Op1328S5 48h47 (FINAL)

FIG. 21

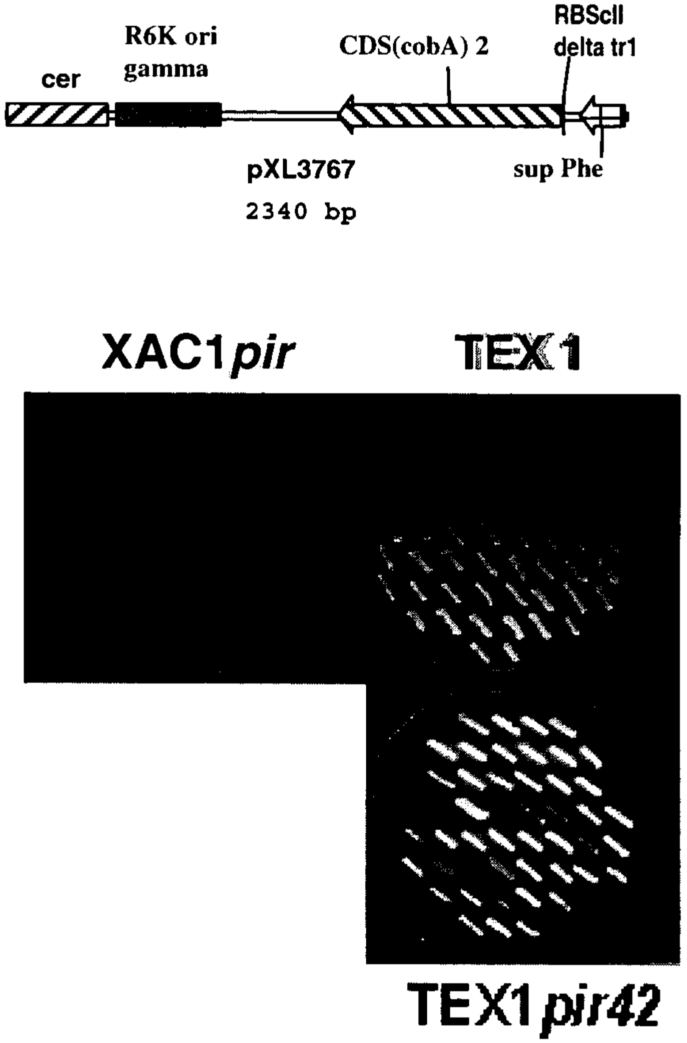

R6K ori
gamma
cer
CDS(cobA) 2
RBScII
delta tr1
pXL3767
2340 bp
sup Phe
XAC1*pir*
TEX1
TEX1*pir42*

FIG. 22

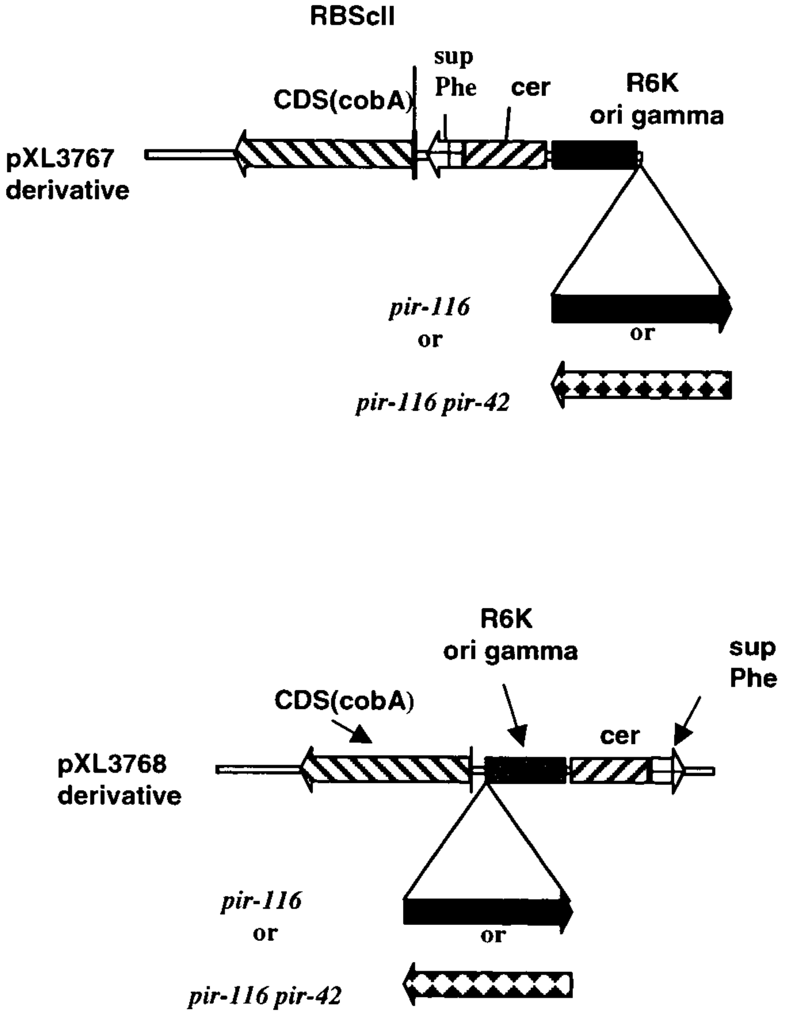

RBScII
sup
Phe
CDS(cobA)
cer
R6K
ori gamma
pXL3767
derivative
pir-116
or
or
pir-116 pir-42
R6K
ori gamma
sup
Phe
CDS(cobA)
cer
pXL3768
derivative
pir-116
or
or
pir-116 pir-42

FIG. 23

pXL 3830

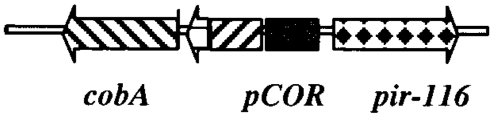


FIG. 24

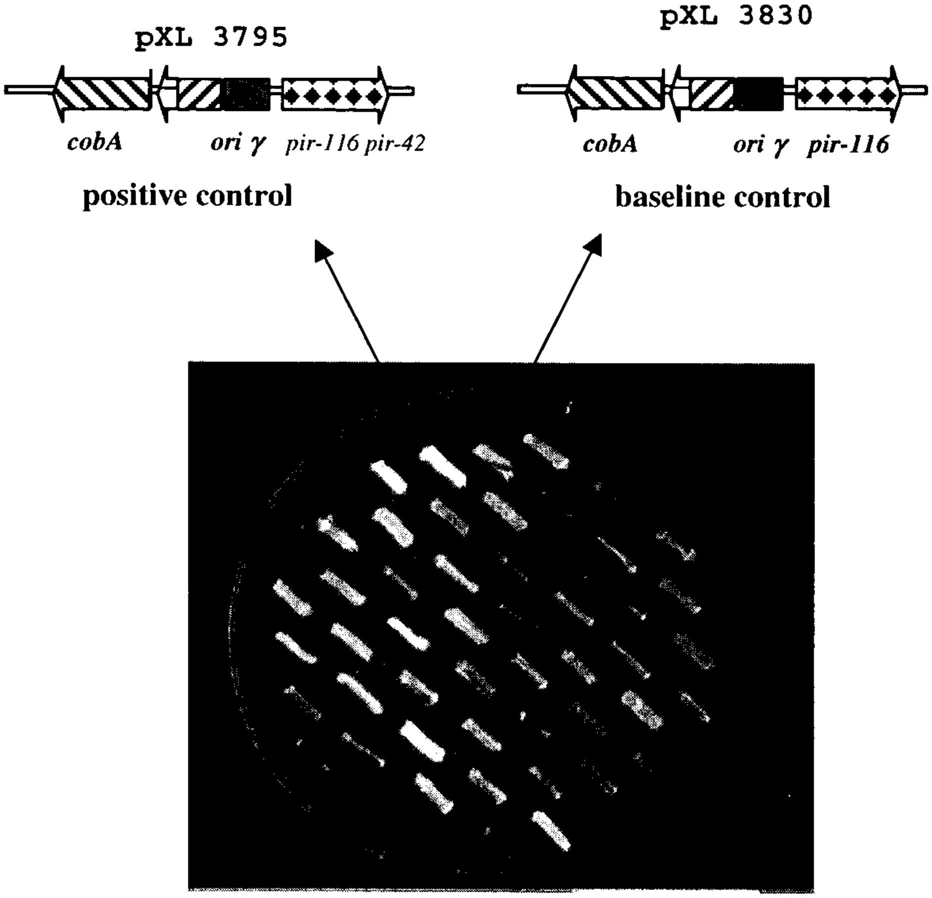

pXL 3795
cobA
ori γ
pir-116 pir-42
positive control
pXL 3830
cobA
ori γ
pir-116
baseline control

FIG. 25

Plasmids carrying	relative copy number	multimers
pir116	1	**
pir116-pir42	4 to 6	***
100B	4 to 5	*
114C	3 to 5	*
201C	5 to 9	***

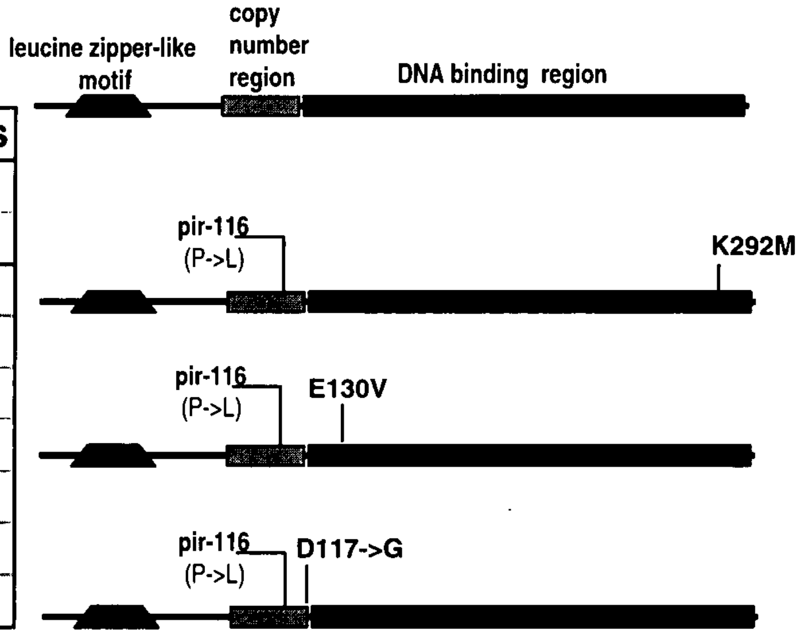


FIG. 26

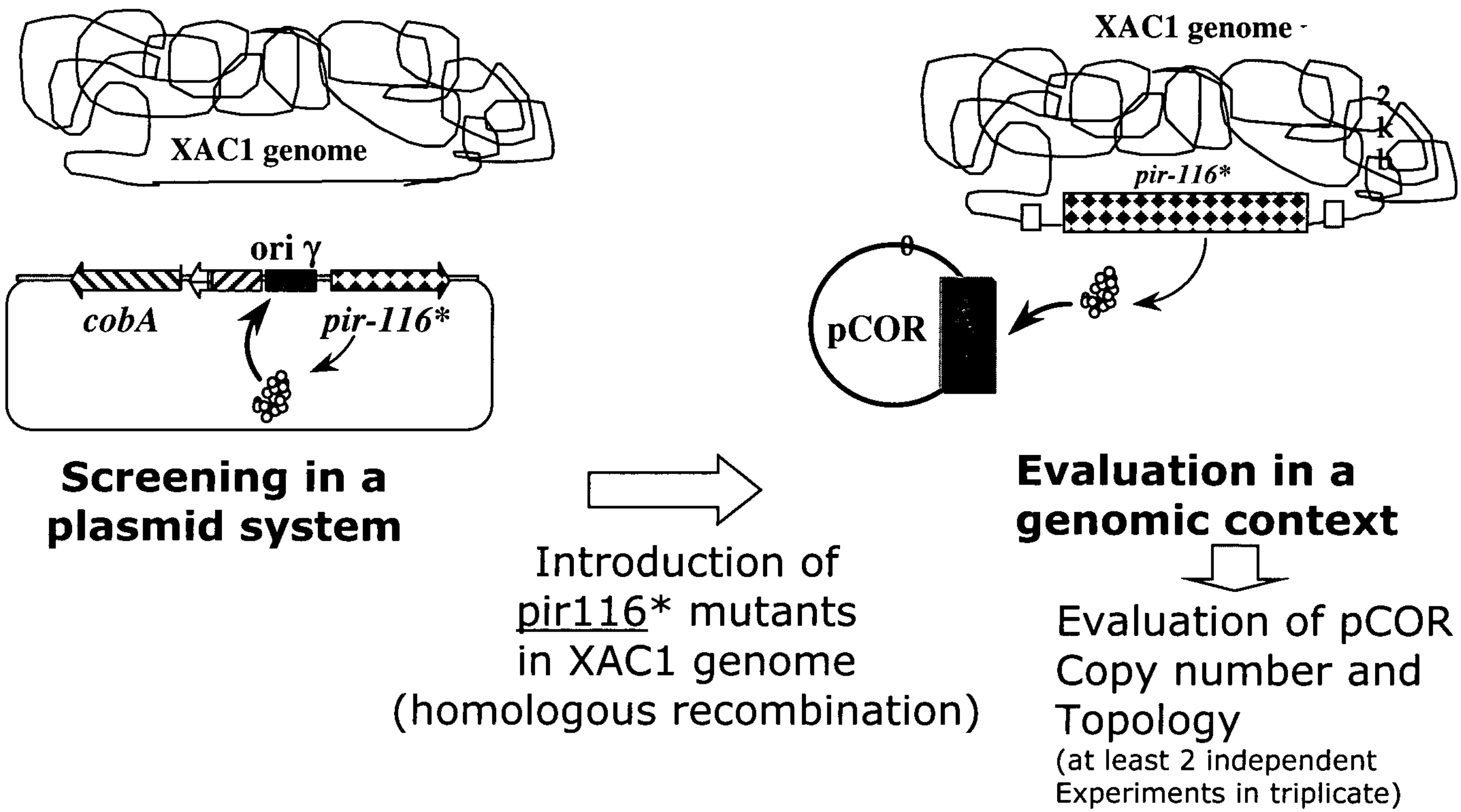

XAC1 genome
ori γ
cobA
pir-116*
Screening in a plasmid system
Introduction of pir116* mutants in XAC1 genome (homologous recombination)
XAC1 genome
2kb
pir-116*
pCOR
Evaluation in a genomic context
Evaluation of pCOR Copy number and Topology
(at least 2 independent Experiments in triplicate)

FIG. 27

pXL3179= NV1FGF (2.4 kb)

E. coli STRAINS	pXL13179 Copy Number	Multimers
XAC1pir116	1	none
TEX1pir42	3 to 4	**
100B1	1.5 to 2	none
114C2	1.5 to 2	**
201C	2 to3	****

FIG. 28

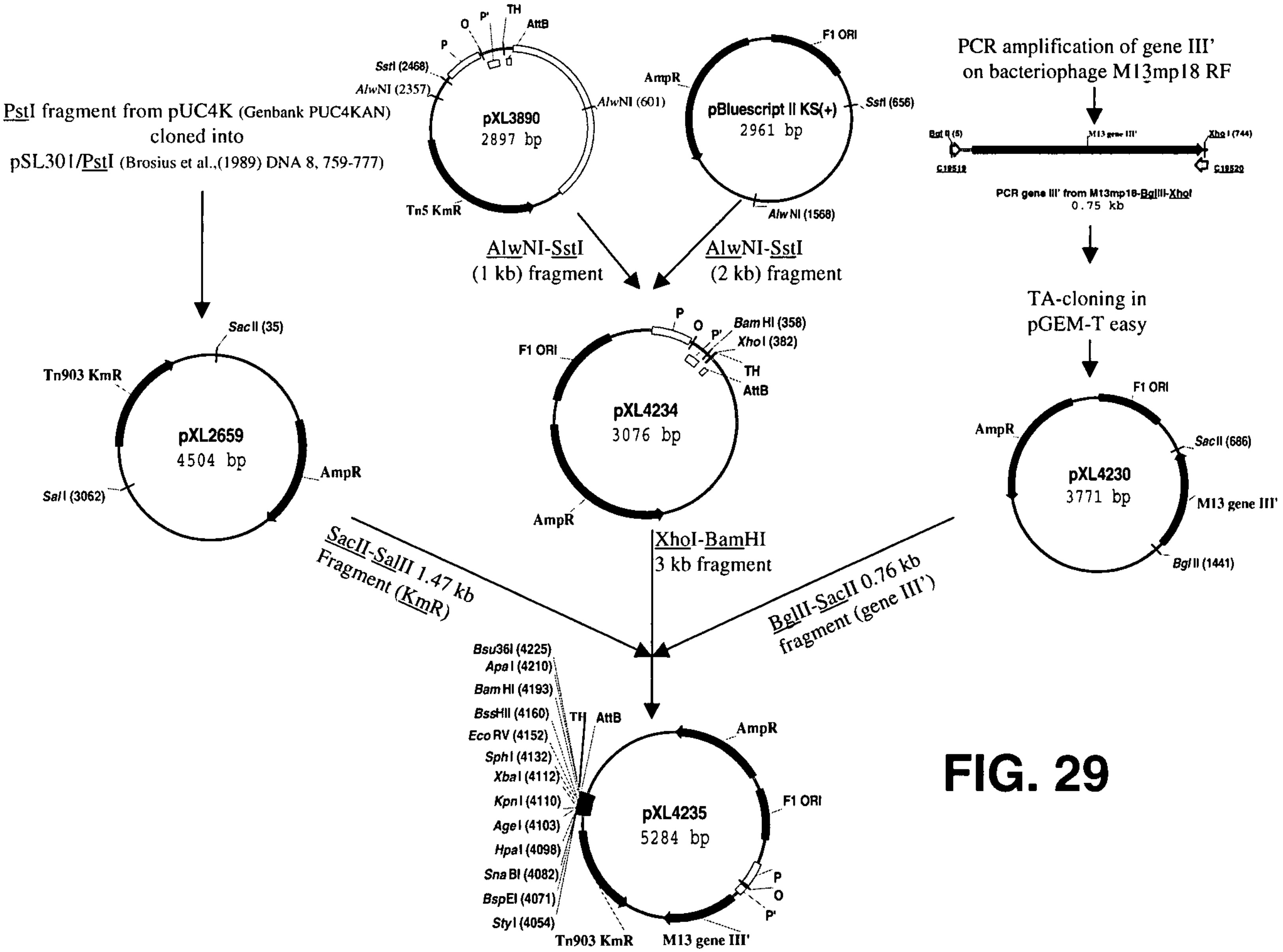

PstI fragment from pUC4K (Genbank PUC4KAN)
cloned into
pSL301/PstI (Brosius et al.,(1989) DNA 8, 759-777)
pXL3890
2897 bp
P
O
P'
TH
AttB
SstI (2468)
AlwNI (2357)
AlwNI (601)
Tn5 KmR
pBluescript II KS(+)
2961 bp
F1 ORI
AmpR
SstI (656)
AlwNI (1568)
PCR amplification of gene III'
on bacteriophage M13mp18 RF
BglII (5)
M13 gene III'
XhoI (744)
C19519
C19520
PCR gene III' from M13mp18-BglII-XhoI
0.75 kb
AlwNI-SstI
(1 kb) fragment
AlwNI-SstI
(2 kb) fragment
TA-cloning in
pGEM-T easy
pXL2659
4504 bp
SacII (35)
Tn903 KmR
AmpR
SalI (3062)
pXL4234
3076 bp
P
O
P'
BamHI (358)
XhoI (382)
TH
AttB
F1 ORI
AmpR
pXL4230
3771 bp
F1 ORI
AmpR
SacII (686)
M13 gene III'
BglII (1441)
SacII-SalII 1.47 kb
Fragment (KmR)
XhoI-BamHI
3 kb fragment
BglII-SacII 0.76 kb
fragment (gene III')
pXL4235
5284 bp
Bsu36I (4225)
ApaI (4210)
BamHI (4193)
BssHII (4160)
EcoRV (4152)
SphI (4132)
XbaI (4112)
KpnI (4110)
AgeI (4103)
HpaI (4098)
SnaBI (4082)
BspEI (4071)
StyI (4054)
TH
AttB
AmpR
F1 ORI
P
O
P'
Tn903 KmR
M13 gene III'

FIG. 29

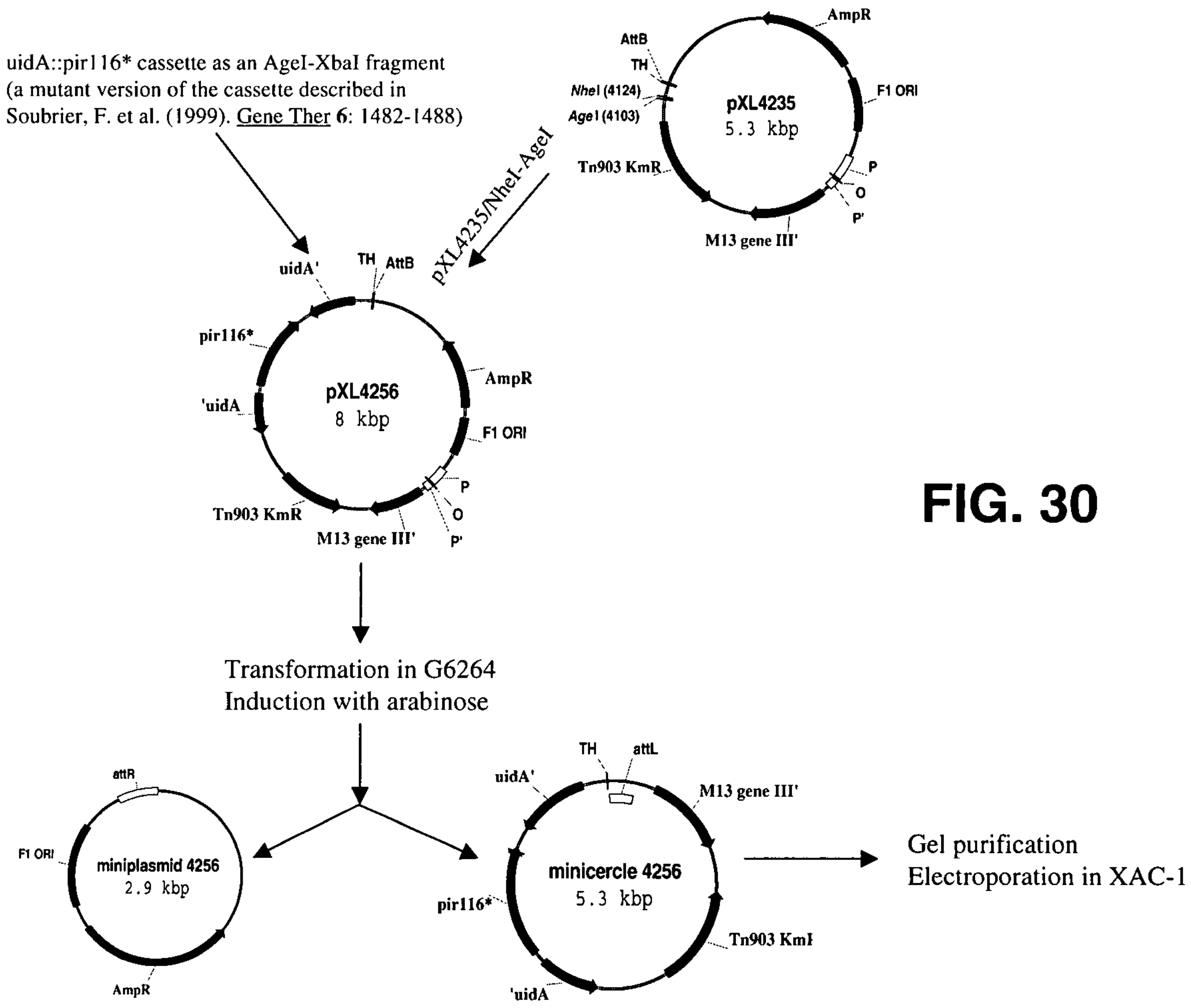
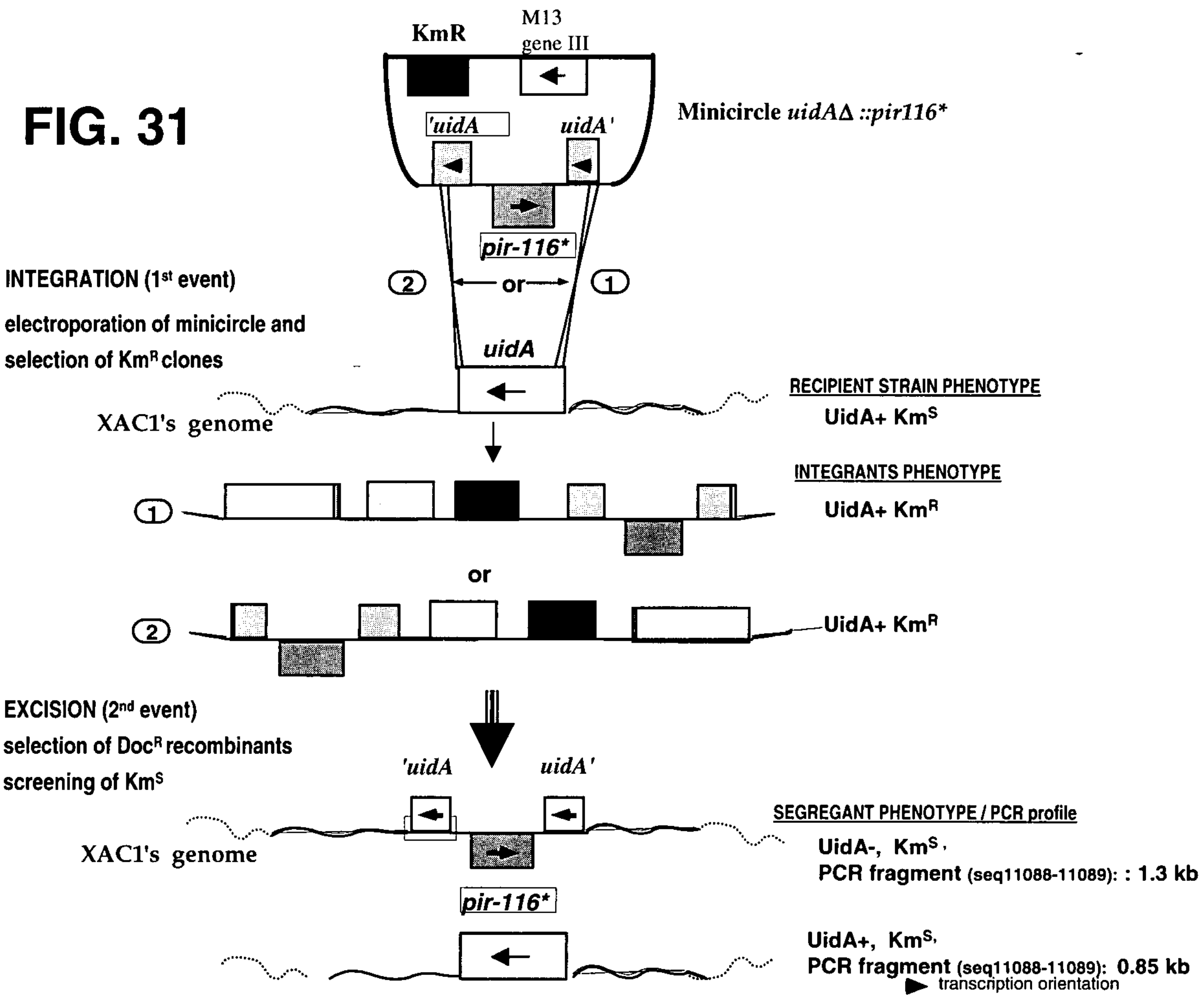

uidA::pir116* cassette as an AgeI-XbaI fragment
(a mutant version of the cassette described in
Soubrier, F. et al. (1999). Gene Ther 6: 1482-1488)
pXL4235/NheI-AgeI
AttB
TH
NheI (4124)
AgeI (4103)
Tn903 KmR
pXL4235
5.3 kbp
AmpR
F1 ORI
P
O
P'
M13 gene III'
uidA'
TH
AttB
pir116*
'uidA
pXL4256
8 kbp
AmpR
F1 ORI
P
O
P'
Tn903 KmR
M13 gene III'
Transformation in G6264
Induction with arabinose
attR
F1 ORI
miniplasmid 4256
2.9 kbp
AmpR
TH
attL
uidA'
M13 gene III'
pir116*
minicercle 4256
5.3 kbp
Tn903 KmI
'uidA
Gel purification
Electroporation in XAC-1

FIG. 30

FIG. 31


KmR
M13
gene III
Minicircle *uidA*Δ *::pir116**
'uidA
uidA'
pir-116*
INTEGRATION (1st event)
electroporation of minicircle and
selection of Km^R clones
②
or
①
uidA
XAC1's genome
RECIPIENT STRAIN PHENOTYPE
UidA+ Km^S
INTEGRANTS PHENOTYPE
①
UidA+ Km^R
or
②
UidA+ Km^R
EXCISION (2nd event)
selection of Doc^R recombinants
screening of Km^S
'uidA
uidA'
XAC1's genome
SEGREGANT PHENOTYPE / PCR profile
UidA-, Km^S,
PCR fragment (seq11088-11089): : 1.3 kb
pir-116*
UidA+, Km^S,
PCR fragment (seq11088-11089): 0.85 kb
▶ transcription orientation

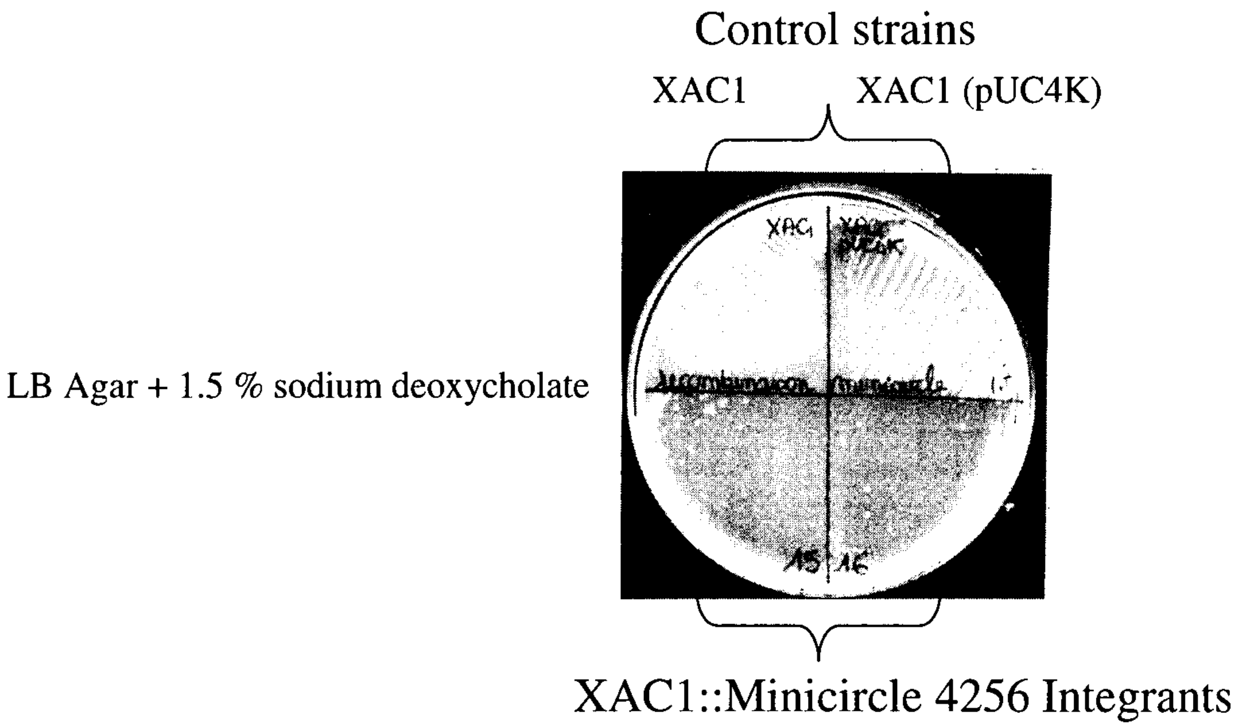
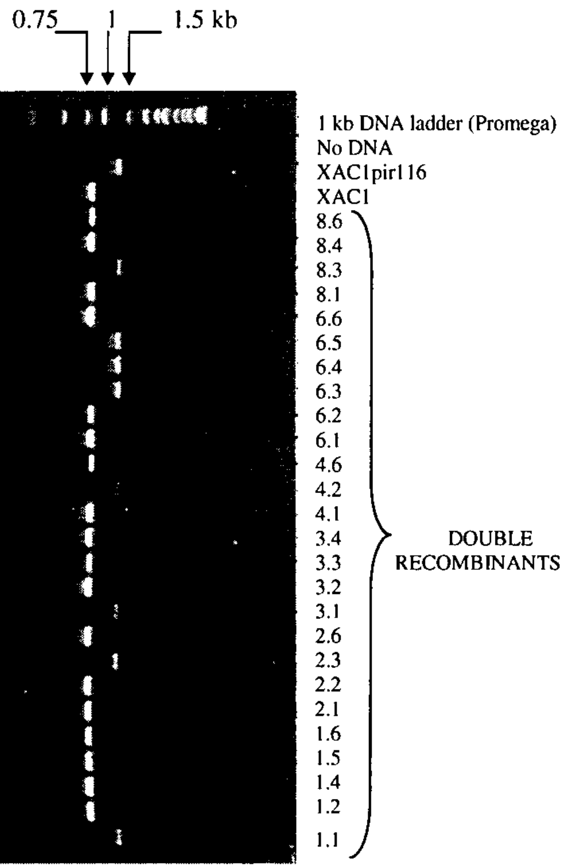
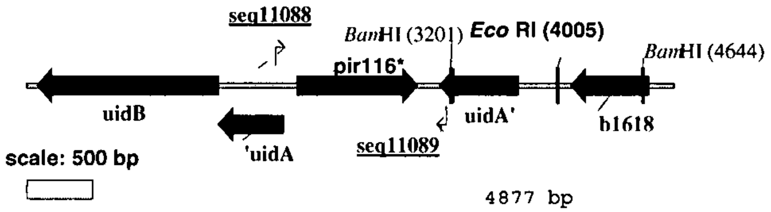

Control strains
XAC1
XAC1 (pUC4K)
LB Agar + 1.5 % sodium deoxycholate
XAC1::Minicircle 4256 Integrants
XAC1
15
16

FIG. 32

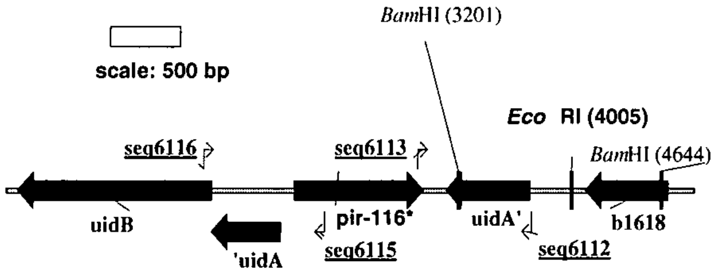


PCR with oligos seq11088 and seq11089

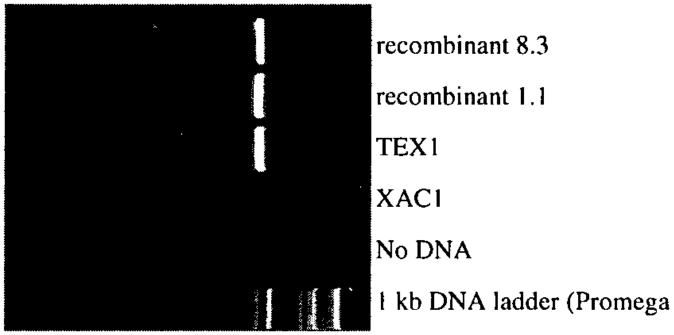


genomic uidAΔ recombinant locus

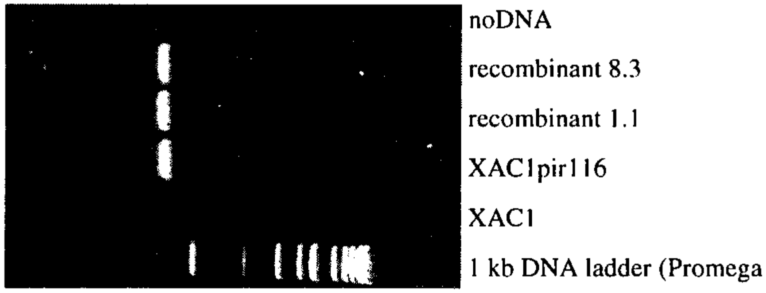
FIG. 33A



genomic uidAΔ recombinant locus 4877 bp



PCR with oligos seq6115 and seq6116



PCR with oligos seq6112 and seq6113

FIG. 33B